# DEVELOPMENT OF METHODS TO MEASURE HEMOGLOBIN ADDUCTS BY GEL ELECTROPHORESIS - PRELIMINARY RESULTS

*Abstract — Chemical adducts formed on blood hemoglobin may be a useful biomarker for assessing human exposures to these compounds. This paper reports preliminary results in the development of methods to measure such adducts that may be generally applicable for a wide variety of chemicals. Male F344/N rats were intraperitoneally injected with $^{14}C$-BaP dissolved in corn oil. Twenty-four hours later, the rats were sacrificed. Blood samples were collected and globin was isolated. Globin protein was then cleaved into peptide fragments using cyanogen bromide and the fragments separated using 2-dimensional gel electrophoresis. The results showed that the adducted $^{14}C$-globin fragments migrated to different areas of the gel than did unadducted fragments. Further research is being conducted to develop methods that will allow quantitation of separated adducted globin fragments from human blood samples without the use of a radiolabel.*

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People are repeatedly exposed to various types of potentially toxic chemicals, particularly in the workplace. Because of this there is a need to accurately determine the internal dose and provide information on the individual's prior exposure history for that compound. One example of a chemical to which people are exposed is benzo(a)pyrene (BaP). This compound is found in cigarette smoke, fossil fuel combustion products, and barbecued foods. When BaP enters the body, it can be metabolically activated to reactive epoxides, which can then nonenzymatically bind covalently to proteins and other macromolecules. Of the numerous blood proteins that can form adducts after chemical exposure, blood hemoglobin (Hb) would be one of the most desirable for use as an exposure monitor. This is because blood samples can be obtained easily, globin is easily isolated and Hb adducts are relatively long-lived in circulating blood (approximately 120 days in man). Previous studies by Segerback,[1] using ethylene and ethylene oxide, have indicated that Hb adducts formed by these compounds can be effectively correlated with human exposure scenarios. Additionally, a number of other reports have been published concerning the use of protein/Hb adducts as biomarkers of exposure.

While it appears that a great deal of research has been done on the use of Hb adducts as a biomarker, the majority of these studies were conducted using compounds containing a radiotracer as the method of detection, or other chemical detection methods that were either not sensitive enough to monitor realistic exposure levels or were not generally applicable for a wide variety of compounds.

The purpose of this study was to develop more sensitive methods for measuring Hb adducts that would be applicable for assessing exposures to a wide variety of compounds found in both the environment and in the workplace. This report describes the preliminary results of techniques to separate and quantitate benzo(a)pyrene-adducted globin from unadducted globin using 2-dimensional gel electrophoresis.

## METHODS

[7,10-$^{14}$C] Benzo(a)pyrene (BaP) was obtained from Amersham at a specific activity of 52 mCi/mmol. Unlabeled BaP (Aldrich) was used to dilute the $^{14}$C-BaP to the desired specific activity.

Male F344/N rats (12-14 wk of age) were injected intraperitoneally (0.5 mL) with $^{14}$C-BaP dissolved in corn oil (10.0 µmol BaP/kg body mass). Rats were sacrificed 24 h later by $CO_2$ asphyxiation and blood samples were collected by cardiac puncture. Globin was isolated from blood samples as originally described by Ascoli et al.[2] The resulting precipitate was washed in pure acetone by centrifugation and then washed with diethylether. The isolated globin was air dried. Approximately 100 mg of the isolated globin was analyzed for $^{14}$C by liquid scintillation spectroscopy to determine the amount of BaP bound per mg globin. The remainder of the globin was stored at -20°C.

SDS polyacrylamide gels (12.5% w/v) were run according to the methods of Davis[3] to determine the purity of the globin (10 µg globin per gel). Molecular weight protein standards were also run on the gels. The globin was then cleaved with CNBr according to the method of Steers et al.[4] This method cleaves proteins at methionine amino acids. A 50-fold molar excess of CNBr over methionine residues, as determined from the amino acid sequence of rat globin alpha and beta chains reported by Hiroyuki et al.,[5] was added. From this amino acid sequence, we would expect to obtain six peptide fragments from CNBr cleavage of globin. The cleaved globin samples were then lyophilized to remove the formic acid and residual CNBr.

CNBr cleaved globin (~ 500 µg) was run on 2-dimensional polyacrylamide gels. The first dimension consisted of a 5% polyacrylamide isoelectric focusing (IEF) gel which was prepared in a 1 mm (id) glass tube and run using Bio-Lyte 3/10 (BioRad Labs) to establish a pH gradient across the gel from pH 3 to pH 10. The sample was loaded onto this gel and run for 7200 volt-hours. Upon completion, the IEF gel was loaded onto the second-dimension slab gel consisting of a 5% polyacrylamide stacking gel (pH 6.8), and a 20% polyacrylamide, 0.5% bis-acrylamide, 10% glycerol resolving gel (pH 8.8). Molecular weight protein standards (BioRad Labs) were also loaded onto this gel. A second, 2-dimensional gel was run in exactly the same manner as described above except that this gel was not stained. Instead, it was cut into 1 cm$^2$ fractions. These fractions were put into scintillation vials containing 1 ml of 10% SDS for 48 h. Scintillation cocktail (10 mL) was then added and the fractions analyzed by liquid scintillation spectroscopy. Fractions containing greater than twice background $^{14}$C radioactivity were correlated with their migration position on the matching gel stained with Coomassie blue. It was expected that adducted CNBr-cleaved globin peptide fragments would migrate differently from corresponding nonadducted globin fragments.

## RESULTS

The injection of rats with $^{14}$C-BaP (10 µmole/kg body weight) resulted in approximately 0.45 pmoles of $^{14}$C-BaP equivalents bound per mg globin. The purity of the isolated globin was assessed by 12.5% SDS polyacrylamide gel electrophoresis. A plot of the log molecular weight vs. relative mobility of molecular weight protein standards showed that the isolated globin migrated with an apparent molecular weight of 16,500 daltons. Densitometric scans of these gels showed that this protein was approximately 95% pure (Fig. 1).

The 2-dimensional gel used to separate adducted from nonadducted CNBr-cleaved globin fragments yielded 9, Coomassie blue-stained spots (Fig. 2). The fractionated 2-dimensional gel analyzed for $^{14}$C gave only 3 radioactive areas which did not co-migrate with any of the stained spots (Fig. 2). Two of them were found to be twice background levels of $^{14}$C, as compared to adjacent gel fractions. The remaining area was found to have 6-times more $^{14}$C than adjacent gel fractions.

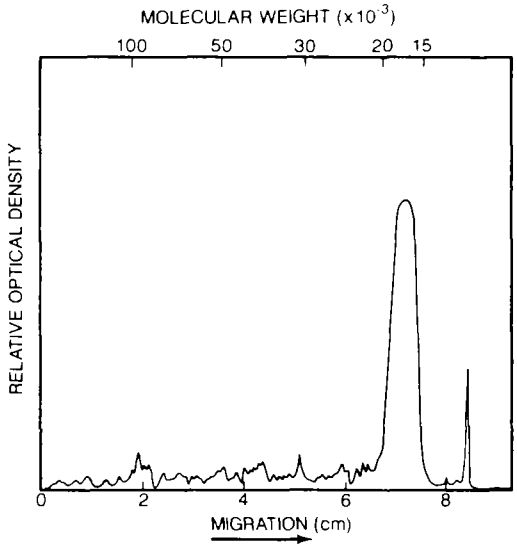


Figure 1. 12.5% SDS polyacrylamide disc gel electrophoretic analysis of globin isolated from animals 24 h after intraperitoneal injection with $^{14}$C-benzo(a)pyrene dissolved in corn oil. Disc gels were stained with Coomassie blue then scanned with a densitometer to give relative optical density units. The arrow indicates the expected migration of globin based on its molecular weight of ~ 16,500 daltons.

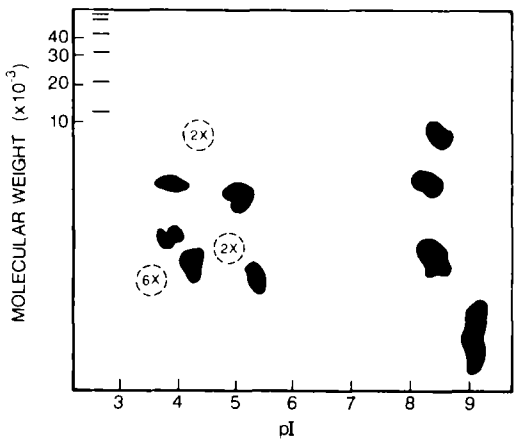


Figure 2. Two-dimensional gel electrophoretic analysis of $^{14}$C-BaP-adducted globin peptide fragments produced by CNBr cleavage. Separation of fragments was based on isoelectric points (pI, horizontally) and molecular weights (vertically). Solid lines indicate fragments stained by Coomassie blue. Dotted lines indicate the migration of $^{14}$C and the amount of radiolabel detected relative to background levels.

## DISCUSSION

Injecting rats with $^{14}$C-BaP and using published methods for isolating blood globin appeared to be an effective technique for obtaining adducted globin in a relatively pure form. Two-dimensional electrophoretic analysis of these samples after CNBr cleavage resulted in a gel with 9 protein spots, as seen by Coomassie blue staining. However, the amino acid sequence of Wistar rat globin would predict that only six fragments would be produced after CNBr cleavage.[5] The discrepancy may be due to the fact that Fisher rat globin has more methionine residues than does Wistar rat globin, or that isozymes of the alpha and/or beta chains exist. Calculations based on the measured degree of binding show that the amount of $^{14}$C-BaP equivalents bound to globin was extremely small (1 adduct/$10^5$ to $10^6$ globin molecules). Thus, only nonadducted, CNBr-cleaved globin fragments were visualized by the stain. Radioanalysis of the fractionated 2-dimensional gel revealed only 3 areas of radioactivity. Since none of these areas of radioactivity corresponded to the stained areas on the gel, they were probably adducted globin peptide fragments that were separated from unadducted fragments.

In summary, the results from this study showed that BaP does form hemoglobin adducts and that BaP-adducted globin can be easily isolated from whole blood in a relatively pure form. Furthermore, it appears that BaP adducts on globin peptide fragments produced by CNBr cleavage can be separated by 2-dimensional gel electrophoresis. Further experimentation is required to verify these results. Additional studies will be conducted to develop methods for labeling these isolated, adducted globin fragments with $^{125}$I after administration of unlabeled BaP to animals. This will allow the quantitation of Hb adducts in people.

## REFERENCES


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